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ORIGINAL ARTICLE

Peripheral chondrosarcoma progression is associated with increased type X collagen and vascularisation

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Abstract Endochondral bone formation requires a cartilage template, known as the growth plate, and vascular invasion, bringing osteoblasts and osteoclasts. Endochondral chondrocytes undergo sequences of cell division, matrix secretion, cell hypertrophy, apoptosis, and matrix calcification/mineralisation. In this study, two critical steps of endochondral bone formation, the deposition of collagen X-rich matrix and blood vessel attraction/invasion, were investigated by immunohistochemistry. Fourteen multiple osteochondromas and six secondary peripheral chondrosarcomas occurring in patients with multiple osteochondromas were studied and compared to epiphyseal growth plate samples. Mutation analysis showed all studied patients (expect one) to harbour a germ-line mutations in either *EXT1* or *EXT2*. Here, we described that homozygous mutations in *EXT1/EXT2*, which are causative for osteochondroma formation, are likely to affect terminal chondrocyte differentiation and vascularisation in the osteocartilaginous interface. Contrastingly, terminal chondrocyte differentiation and vascularisation seem to be unaffected in secondary peripheral chondrosarcoma. In addition, osteochondromas with high vascular density displayed a higher proliferation rate. A similar apoptotic rate was observed in osteochondromas and secondary peripheral chondrosarcomas. Recently, it has been shown that cells with functional *EXT1* and *EXT2* are outnumbering *EXT1/EXT2* mutated cells in secondary peripheral chondrosarcomas. This might explain the increased type X collagen production and blood vessel attraction in these malignant tumours.

Keywords Growth plate · Osteochondroma · Peripheral chondrosarcoma · Type X collagen · Angiogenesis · Bone tumour

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Introduction

Elongation of long bones is a complex process that requires a cartilage template, known as the epiphyseal growth plate, and a strict coordination and synchronisation of cell–cell and cell–matrix signalling events [1]. The key events in this process can be divided into two major sequential morphogenesis phases. The first phase is characterised by the proliferation of the growth plate chondrocytes, which maintains the pool of cells needed for bone lengthening. The second phase is a multistep process characterised by the hypertrophy of proliferating chondrocytes and bone formation. Hypertrophic chondrocytes increase their volume and secrete a specialised extracellular matrix rich in type X collagen [2]. The ossifying collagen X-rich matrix attracts blood vessels and bone precursor cells, allowing bone development [3].

The process of endochondral ossification is also observed in cartilaginous tumours [4]. An active endochondral ossification takes place deep to the cartilage cap of osteochondroma and secondary peripheral chondrosarcoma [5].

Osteochondromas are the most common benign bone tumours at childhood and adolescence [6]. They can occur either as a sporadic lesion or multiple tumours in hereditary multiple osteochondromas syndrome (previously known as hereditary multiple exostoses) [7]. Osteochondromas are caused by mutations in either *EXT1* or *EXT2* genes [7].

They are pedunculated or sessile cartilage-capped bony projections from the metaphyses of endochondral bones adjacent to the growth plate [5]. In less than 1% of patients with sporadic osteochondromas and 1–3% of patients with multiple osteochondromas at the age of 30–60 years, an osteochondroma may eventually transform into a secondary peripheral chondrosarcoma [8].

Whereas osteochondromas have been linked to homozygous mutations in *EXT1* or *EXT2* genes, cells with functional *EXT1* and *EXT2* have been shown to be the predominant subclone in secondary peripheral chondrosarcomas [9].

Mutations in *EXT1* have been demonstrated to impair angiogenesis in mice [10] and to affect endochondral bone formation by reducing type X collagen deposition [11]. Endochondral bone formation has been shown to be affected in the homozygous *dak/ext2* zebrafish mutant [12].

EXT1 and *EXT2* genes encode enzymes that catalyse the biosynthesis of heparan sulphate. Heparan sulphate is a key component of the extracellular matrix acting as co-receptors for signalling molecules, including vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), and others [13, 14]. The impact of mutations in *EXT1* or *EXT2* genes with regards to production of a collagen X-rich matrix and attraction of blood vessels in osteochondromas and secondary peripheral chondrosarcomas has not been investigated so far.

Here, we describe that terminal chondrocyte differentiation and vascularisation are affected in osteochondromas. Contrastingly, these two critical steps of endochondral bone formation seem to be unaffected in secondary peripheral chondrosarcomas.

Materials and methods

Patient material

Paraffin-embedded tissues from 14 osteochondromas and 6 low-grade secondary peripheral chondrosarcomas from patients with multiple osteochondromas, collected between 1991 and 2008, were retrieved from the files of the Leiden University Medical Centre (Table 1). One patient had two tumours from distinct location included in this study. For comparison, paraffin-embedded epiphyseal growth plates ($n=3$) were obtained from orthopaedic resections for pathological conditions not related to osteochondroma or chondrosarcoma. Clinical information and the thickness of the cartilage cap were obtained from pathology/radiology reports. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines in "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Deparaffinised sections were treated with testicular hyaluronidase (2 mg/ml in 0.1-M Tris saline, pH 5.0; Sigma-Aldrich, St Louis, MO, USA, 60 min at 37°C) and proteinase K (5 μl/ml in 0.1-M Tris-buffered saline, pH 5.0; DakoCytomation, Carpinteria, CA, USA) followed by overnight incubation at 4°C with monoclonal antibody to collagen X (clone X53, 1:100, Quartett, Berlin, Germany), as previously described [4, 15]. Monoclonal antibody to CD31 (clone JC70A, 1:10,000, DakoCytomation, Glostrup, Denmark), cleaved caspase-3 (clone 5A1E, 1:100, Cell Signaling Technology, Danvas, MA, USA), and Ki-67 (clone MIB-1, 1:100, Dako, Glostrup, Germany) were used as earlier described [16–18]. The thickness of collagen X-rich matrix was evaluated jointly by two observers (C.E.A. and S.R.). The thickest collagen X stained area was identified in each section and measured by an eye-piece graticule, as described in Fig. 1a. The apoptotic rate was defined as the number of apoptotic cells (positive for caspase-3) out of the total number of nucleated cells. The proliferation rate was determined by counting the numbers of Ki-67-positive cells.

Estimating vascular density by Chalkley counting

The Chalkley counting method has been previously described in detail [19]. Briefly, the three most vascular areas (hot spots) of each section stained with CD31 were selected and analysed (Fig. 1b). Each vascular hotspot was viewed at×200 magnification, and a 25-point Chalkley graticule was applied and oriented to permit the maximum number of points to hit in or within the CD31 stained blood vessels [19, 20]. The Chalkley count for each section was taken as the mean value of the three counts and performed by two observers independently (C.E.A. and J.V.M.G.B.).

Mutation analysis

Eleven patients were screened by direct sequencing for DNA mutations in the coding sequence of *EXT1* or *EXT2* genes [21]. After informed consent, DNA was isolated from resected tumours. If no alterations were found, a multiplex ligation-dependent probe amplification assay designed for *EXT1* and *EXT2* genes was performed to identify possible large deletions, as previously described [22]. Mutation analysis from six patients has been previously reported [15, 23, 24] (Table 1).

Statistical analysis

Results are expressed as the means with a measure of variability (standard deviation, S.D.). Statistical significance was calculated by one-way analysis of variance with

Table 1 Clinical information of patients with osteochondroma and secondary peripheral chondrosarcoma

Case #	Sample	Age (years)	Gender	Location	*EXT* mutation analysis[a] (type of mutation)	Cartilage cap thickness (mm)
L-741	GP	2	M	Femur	–	–
L-996	GP	8	M	Femur	–	–
L-1142	GP	12	F	Tibia	–	–
L-1234	GP	8	F	Femur	–	–
L-298	OC	24	M	Femur	*EXT1* exon 3: c.1121 G > A, p.W374X (ns)[b]	6
L-317	OC	31	M	Tibia	None in *EXT1* or *EXT2*[b]	4
L-332	OC	25	M	Femur	None in *EXT1* or *EXT2*[b]	5.5
L-523	OC	20	M	Tibia	*EXT1* del codon 235–239 (del Pro-Leu-Phe-Ser-Lys)[b]	5
L-524	OC	26	M	Tibia	*EXT1* codon 164 GAC > CAC (Asp > His), exon 1: c.1212 G > C, p. D164H[b]	4.5
L-722	OC	15	F	Radius	*EXT2* IVS7+1 G > A[b]	1.5
L-726	OC	6	M	Rib	*EXT2* IVS7+1 G > A	6
L-841	OC	14	F	Femur	*EXT2* deletion exon 6+7 (del)[b]	8
L-1094	OC	6	M	Femur	*EXT2* del exon 6+7	3
L-1143	OC	39	M	Femur	NA	2
L-2029	OC	23	F	Femur	*EXT2* exon 7: IVS7+1 G > A	3.5
L-2069	OC	11	M	Tibia	*EXT1* exon 1 c.538_539delAG, p.Ser180fsX7[b]	5.5
L-2160	OC	48	F	Scapula	*EXT1* exon1 c.538_539delAG, p.Ser180fsX17	6
L-2350	OC	53	F	Femur	*EXT2* exon 7: IVS7+1 G>A	1
L-951	PCH	24	M	Fibula	NA	8
L-2117	PCH	37	M	Humerus	*EXT1* exon 2: c.1056+1 G>A	12
L-114	PCH	39	M	Femur	*EXT2* exon 5: 764 T>C, L255P (uv)[b]	12
L-578	PCH	22	F	Femur	*EXT2* deletion exon 6+7 (del)	11
L-2254	PCH	33	F	Pelvis	*EXT2* exon 6 c.980delG, p.Gly327AlafsX5	8
L-2372	PCH	54	M	Rib	NA	13

GP epiphyseal growth plate, *OC* osteochondroma, *PCH* low grade secondary peripheral chondrosarcoma, *NA* not analysed

[a] Mutation nomenclature was according to the Nomenclature Working Group [30]; *ns* non-sense, *del* deletion, *uv* unclassified variant, *fs* frame shift, *pm* polymorphism

[b] Results of mutation previously reported [15, 23, 24]

Bonferroni's multiple comparison tests. Spearman's rank correlation coefficient was calculated to verify statistical dependence between two variables. Both tests were performed using the SPSS 16.0 software package (IBM, Somers, NY, USA). P values <0.05 were considered significant.

Results

Collagen X-rich matrix in peripheral cartilaginous tumours

The epiphyseal growth plate is organised in columns of stacked chondrocytes (Fig. 2a). The interface between cartilaginous and osseous parts in the growth plate (osteocartilaginous interface) was well defined and formed by hypertrophic chondrocytes immersed in a strong stained collagen X-rich matrix (Fig. 2d). In the growth plate, the collagen X-rich matrix was restricted to the hypertrophic zone and had, on average, 0.8 (± 0.47) mm thickness.

Osteochondromas showed less structured tissue organisation. The osteocartilaginous interface was also less well defined (Fig. 2b). The collagen X-rich matrix was restricted to the hypertrophic zone and had, on average, 1.5 (± 1.48) mm thickness. Additionally, in osteochondroma, not all chondrocytes with a hypertrophic morphology expressed type X collagen (Fig. 2e), and when type X collagen was present, the staining was mainly seen around the chondrocytes and not across the matrix (Fig. 2e). In osteochondromas, no correlation was found between the age of the patient and the thickness of the collagen X-rich matrix (Spearman's coefficient =0.25).

Secondary peripheral chondrosarcomas showed no structured tissue organisation (Fig. 2c) and had an average cap thickness of 10 mm. Secondary peripheral

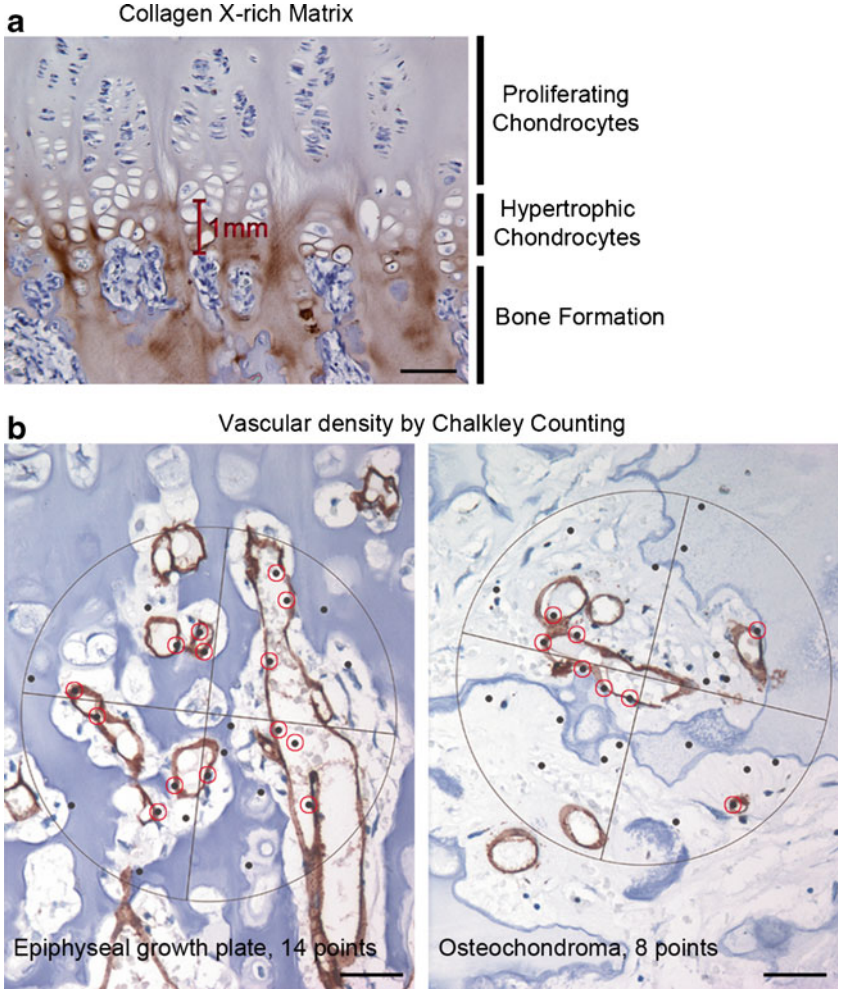

Fig. 1 Collagen X-rich matrix and vascular density by Chalkley counting. Collagen X-rich matrix produced by hypertrophic chondrocytes was measured from the beginning to the end of the hypertrophic zone (**a**). Chalkley count reflects the number of grid points that hit CD31 stained vessels (*red circles*) (**b**). It is more of an estimate of the relative area than a true vessel count (**a** *scale bar* 10 μm; **b** *scale bars* 5 μm)

chondrosarcomas showed a thick, uniformly and strongly stained collagen X-rich matrix (Fig. 2f) with an average 6.5 (± 3.66) mm thickness. Type X collagen expression in secondary peripheral chondrosarcomas was not restricted to the osteocartilaginous interface but was also detected throughout the extracellular tumour matrix and close to the perichondrium (data not shown). In secondary peripheral chondrosarcomas, no correlation was found between the age of the patient and the thickness of the collagen X-rich matrix (Spearman's coefficient =0.01).

In osteochondromas and secondary peripheral chondrosarcomas, the thickness of the collagen X-rich matrix correlated with the thickness of the cartilage cap (Spearman's coefficient =0.63) (Fig. 2g).

Similar apoptotic rates were observed in osteochondromas and secondary peripheral chondrosarcomas and had values of 35 (± 0.16) % vs. 36 (± 0.15) %, respectively (P= 0.847).

Vascular density in the ossification area

Invasion of blood vessels is a critical step towards ossification of the collagen X-rich matrix. Strong CD31 staining was observed along the cell membrane of endothelial cells in the epiphyseal growth plate, osteochondroma, and secondary peripheral chondrosarcoma. The median CD31 vascular density in the growth plate osteocartilaginous interface was significantly higher than in osteochondroma, with a value of 10.8 (± 0.83) vs. 6.1 (± 1.79), respectively (P= 0.007) (Fig. 3a, b, d). Interestingly, no differences in vascularisation of the osteocartilaginous interface were observed between the growth plate and secondary peripheral chondrosarcoma; a value of 10.8 (± 0.83) vs. 10 (± 0.70) was, respectively, found (P= 0.464) (Fig. 3a, c, d). Moreover, an irregular distribution of blood vessels was identified in the osteocartilaginous interface of osteochondromas and peripheral chondrosarcomas, but not in the epiphyseal growth plates.

In osteochondromas and secondary peripheral chondrosarcomas, no association between the age of the patient and vascular density was found (Spearman's rho correlation coefficient =0.39 and 0.27, respectively).

In osteochondromas, the proliferation rate ranged from 0–24% in 9 cases to 25–49% in 5 cases. The cases with a higher proliferation rate (25–49%) showed a higher

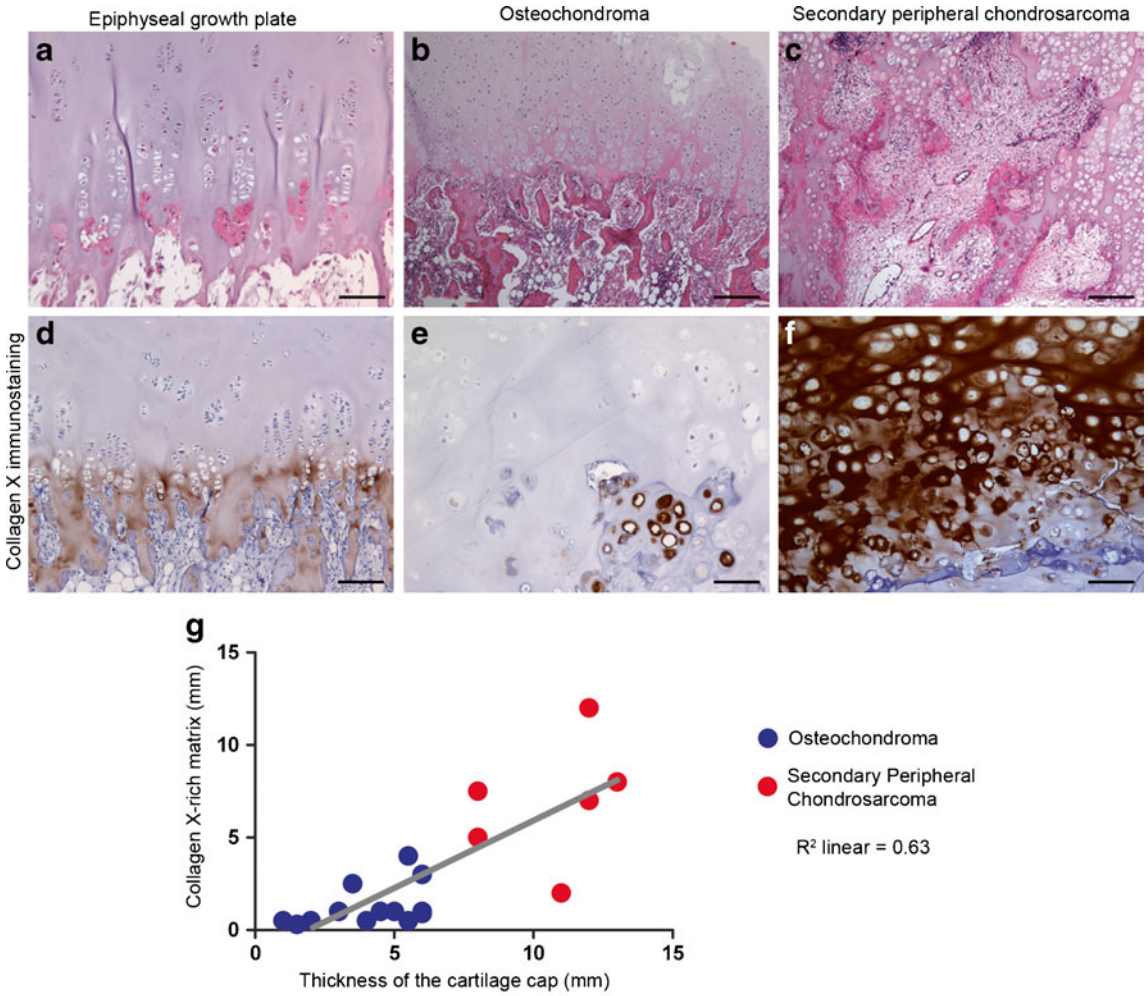

Fig. 2 Collagen X-rich matrix. The epiphyseal growth plate is organised in columns of stacked chondrocytes (**a**). Osteochondromas show less structured organisation (**b**), and secondary peripheral chondrosarcomas (**c**) display no clear organisation. Hypertrophic chondrocytes secrete a collagen X-rich matrix, which, ultimately, is replaced by bone (**d**). In osteochondromas, chondrocytes with a hypertrophic morphology are not always secreting collagen X (**e**). In secondary peripheral chondrosarcoma, a thick layer of collagen X-rich matrix is observed (**f**). In osteochondromas and secondary peripheral chondrosarcomas, the thickness of the cartilage cap correlates with the thickness of collagen X-rich matrix (**g**) (*scale bars* 10 μm)

vascular density when compared to the cases with a lower proliferation rate (0–24%); a value of 8 (± 0.83) vs. 5.2 (± 1.58) was, respectively, found ($P = 0.008$).

Mutation analysis

Fifteen of 16 patients with multiple osteochondromas demonstrated a mutation in *EXT1* or *EXT2* (Table 1). One patient, from whom two distinct tumours were studied (L-317 and L-332), displayed no mutations in *EXT1* or *EXT2*. Additionally, no correlation was found between mutation type and (a) either tumour type ($P=0.564$) (b) or the thickness of the collagen X-rich matrix ($P=0.564$) (c) or vascular density ($P=0.556$).

Discussion

In this study, two crucial steps of endochondral ossification, formation of a collagen X-rich matrix and invasion/attraction of blood vessels, were assessed and compared in the epiphyseal growth plate and peripheral cartilaginous tumours (osteochondroma and secondary peripheral chondrosarcoma). The protein expression level of type X collagen in human osteochondroma was shown to be similar to the one found in human epiphyseal growth plate [4]. Interestingly, in the *EXT1* heterozygous mutant mice, in situ hybridisation studies have demonstrated that the mRNA expression of type X collagen is reduced in the growth plate as compared to wild-type mice [11, 25]. In

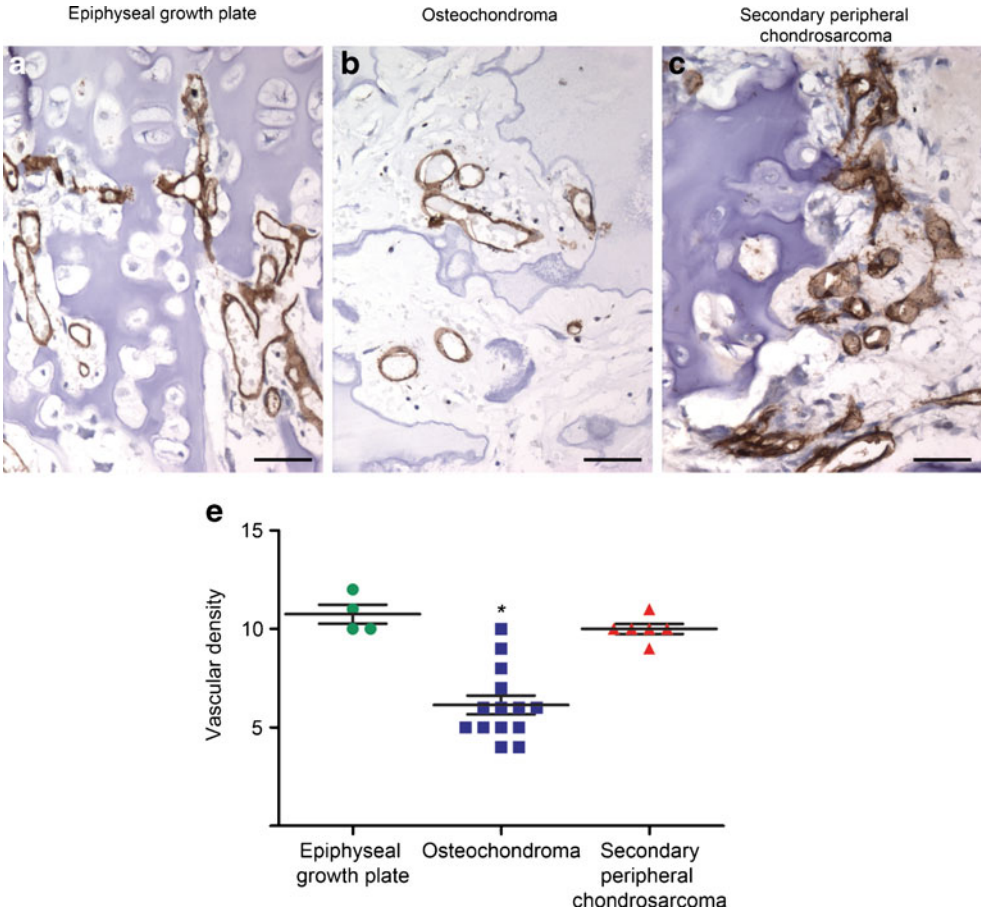

Fig. 3 Vascular density in the ossification zone. The epiphyseal growth plate (**a**, **d**) and secondary peripheral chondrosarcoma (**c**, **d**) show similar vascular density. Osteochondroma displays low vascular density (**b**, **d**) (*asterisk* indicates $P<0.05$; *scale bars* 5 μm)

addition, endochondral bone formation has been shown to be affected in the homozygous *dak/ext2* zebrafish mutant [12]. The reduced type X collagen expression in the *EXT1* mutant mice and the delay in endochondral ossification in the homozygous *dak/ext2* zebrafish mutant suggest that *EXT1* and *EXT2* genes play a role in terminal chondrocyte differentiation and the replacement of hypertrophic chondrocytes by bone.

Here, we describe that not all of the osteochondroma cells undergo terminal differentiation. These cells, although displaying a hypertrophic phenotype, do not express type X collagen (Fig. 2e). This may suggest that mutations in *EXT1* or *EXT2* genes may affect terminal chondrocyte differentiation in humans. Interestingly, progression of osteochondroma towards secondary peripheral chondrosarcoma showed an increased production of type X collagen. Therefore, the secondary peripheral chondrosarcoma cells expressing abundant type X collagen indicated that they have undergone terminal chondrocyte differentiation.

Osteochondromas and secondary peripheral chondrosarcoma had a similar apoptotic rate. This indicates that, although secondary peripheral chondrosarcoma cells undergo terminal differentiation, the differentiation process does not induce apoptosis. Hence, low cell death seems to contribute to the relatively rapid growth of peripheral chondrosarcoma.

Several pro-angiogenic growth factors, such as FGF-2 and VEGFs, bind to heparan sulphate proteoglycans [26]. Heparan sulphate proteoglycans have been shown to regulate the distribution of these signalling molecules throughout the extracellular matrix and their receptor binding affinity [27]. Syndecan-2 is a cell surface heparan sulphate proteoglycan [26]. Syndecan-2 has been demonstrated to bind to VEGF, thereby regulating the distribution of VEGF nearby its receptor [28]. Interestingly, in osteochondromas, syndecan-2 is no longer located in the plasma membrane, but is shown to be accumulated in the Golgi apparatus [24]. The abnormal intracellular location of syndecan-2 may affect the distribution of VEGF, explaining the low efficiency of the osteochondroma cells in attracting blood vessels. Osteochondromas with high vascular density in the osteocartilaginous interface displayed a higher proliferation rate. This might suggest that proliferation of tumour cells is associated with increased vascularisation in the osteocartilaginous interface. Interestingly, secondary peripheral chondrosarcomas showed

similar efficiency in attracting blood vessels as the epiphyseal growth plate.

We have recently shown that cells with functional *EXT1* and *EXT2* genes are the predominant subclone in secondary peripheral chondrosarcomas [9]. Therefore, the increased type X collagen deposition and blood vessel attraction in the osteocartilaginous interface of secondary peripheral chondrosarcoma are possibly associated with cells with functional *EXT1* and *EXT2* genes. In addition, collagen X-rich matrix formation and vascularisation might be useful prognostic markers of neoplastic transformation of an osteochondroma, but further validation is required before they can be proposed as a routine and as a reliable diagnostic tool.

In conclusion, we show that terminal chondrocyte differentiation and vascularisation are affected in osteochondromas, suggesting that *EXT1* and *EXT2* genes are critical for endochondral bone formation. Terminal chondrocyte differentiation and vascularisation seem to be unaffected in secondary peripheral chondrosarcomas, which might be associated with the presence of cells with functional *EXT1* and *EXT2* genes.

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Conflict of interest The authors declare that they have no conflict of interest.

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